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Screening of miRNAs associated with lymph node metastasis in Her-2-positive breast cancer and their relationship with prognosis*#

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Abstract: The aim of this study was to identify some biomarkers for predicting lymph node metastasis and prognosis of human epidermal growth factor receptor 2 (Her-2)-positive breast cancer (BC). We analyzed correlations between microRNAs (miRNAs) and the prognosis of patients with BC based on data collected from The Cancer Genome Atlas (TCGA) database. The expression levels of miR-455, miR-143, and miR-99a were measured in clinical samples of Her-2-positive BC patients with different degrees of lymph node metastasis. We investigated the impacts of overexpressed miR-455 on the proliferation and invasiveness of MDA-MB-453 cells and measured its effects on the expression of long non-coding RNA (lncRNA) metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*) by quantitative real-time polymerase chain reaction (qRT-PCR). The expression of miR-455 was significantly and positively correlated to the prognosis and overall survival (OS) of the BC (P=0.028), according to TCGA information. The expression level of miR-455 was positively correlated with OS and relapse-free survival (RFS) of patients with Her-2-positive BC, and was negatively correlated with the number of metastatic lymph nodes (P<0.05). Transwell assay suggested that MDA-MB-453 cells became much less invasive (P<0.01) after being transfected with miR-455 mimics. During the qRT-PCR, the expression level of *MALAT1* declined significantly after transfection (P<0.01). Overexpressed miR-455 significantly inhibited the proliferation and migration of MDA-MB-453 cells and the expression of *MALAT1*. We conclude that miR-455 may be a useful potential biomarker for forecasting lymph node metastasis and the prognosis of Her-2-positive BC patients. miR-455 may play an important role in lymph node metastasis of BC by interacting with *MALAT1*.

Key words: Breast cancer (BC); Human epidermal growth factor receptor 2 (Her-2); Lymphatic metastasis; MicroRNA (miRNA); Metastasis-associated lung adenocarcinoma transcript 1 *(MALAT1)*

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1 Introduction

For breast cancer (BC), metastasis is most often and most easily detected in regional lymph nodes at the early stage, then in distant organs such as the lungs, bones, liver, and brain. The frequency and location of BC metastasis are closely associated with the type of primary tumors (Weigelt et al., 2005; Zhao et al., 2017). However, no measures have been found to be effective for forecasting and intervening in BC metastasis.

Molecular subtypes largely reflect the clinical characteristics and prognosis of BC. Therefore, distinguishing different molecular subtypes of BC is helpful in the search for more effective therapies (Zhang and Liu, 2008). Of all the molecular subtypes,

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prognosis of human epidermal growth factor receptor 2 (Her-2)-positive BC is only slightly better than that of triple negative breast cancer (TNBC). Her-2-positive BC is associated with a considerable proportion of brain metastases (Aversa et al., 2014). Although Herceptin (trastuzumab) significantly improves the prognosis of Her-2-positive BC, for BC where metastasis has occurred (especially brain metastasis), the combined use of Herceptin and lapatinib in targeted therapies contributes to a considerably higher survival rate than the use of Herceptin alone (Yap et al., 2012). Therefore, there is an urgent need to find biomarkers for identifying the risks of tumor metastasis at the early stage, to customize more suitable personalized therapies for patients with Her-2-positive BC.

Some studies regarding non-coding RNA (ncRNA), particularly microRNAs (miRNAs), have brought new hope for diagnosing and treating numerous types of malignancies (Wang et al., 2015). miRNAs mainly play roles in mediating the degradation of target message RNAs (mRNAs) or inhibiting mRNA translation (McGuire et al., 2015). Studies based on high-throughput sequencing have proven that changes in the expression of numerous miRNA transcripts are correlated with metastasis of BC (Volinia and Croce, 2013; Markou et al., 2014). More importantly, metastasis of BC is considerably suppressed by recovering the expression of some miRNAs (Tavazoie et al., 2008). In addition, the expression levels of miRNAs differ significantly among molecular subtypes of BC. Hence, the identification of one or more miRNAs for a certain type of BC and clarifying its relationship to the development and metastasis of the BC can lead to the discovery of biomarkers related to metastasis, which may improve early diagnosis and treatment.

The typical molecular mechanism underlying the role of miRNAs is as follows: miRNAs suppress translation of a target mRNA or promote its degradation by combination with its 3'-untranslated region (3'-UTR) or open reading frame (ORF) through complementary base pairing inside cells (Bartel, 2004). Hence, miRNAs might regulate metastasis of BC by targeting some metastasis suppressor genes. Recent research has suggested that apart from binding with the target mRNA, some miRNAs might also interact with long non-coding RNAs (lncRNAs) to inhibit the binding between the miRNA and its target mRNA, thus activating the target mRNA. Therefore, these lncRNAs and target mRNAs are competing endogenous RNAs (ceRNAs) (Salmena et al., 2011).

In our early studies, the expression level of highly expressed lncRNA metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*) was closely correlated with lymph node metastasis of Her-2-positive BC. *MALAT1* knockdown at a cellular level significantly inhibits the proliferation and invasiveness of BC cells (Zhang et al., 2018), which suggests that *MALAT1* promotes lymph node metastasis of BC. As a ceRNA, *MALAT1* has proved to be effective for regulating the expression of genes related to tumor metastasis such as enhancer of zeste homolog 2 (*EZH2*) (Hirata et al., 2015), matrix metalloproteinase-14 (*MMP14*) and *Snail* (Luan et al., 2016). However, which miRNAs regulate metastasis of BC or whether their regulation is based on their interactions with *MALAT1* has not been clearly reported.

In this study, miRNA sequencing (miR-seq) data for BC were downloaded from The Cancer Genome Atlas (TCGA) database (https://tcga-data.nci.nih.gov/tcga) and analyzed, and a literature search was conducted. The expression levels of miRNAs and lncRNA *MALAT1* in clinical samples were measured via quantitative real-time polymerase chain reaction (qRT-PCR).

2 Materials and methods

2.1 Results of miR-seq

Information about patients with BC was downloaded from the TCGA database on Dec. 16, 2016. Ultimately, miR-seq results of 1181 samples were obtained. They covered raw data on miR-seq of 1077 samples of BC tissues and 104 samples of paracancerous tissues as well as corresponding clinical information of 1078 samples.

2.2 Screening of miRNAs related to lymph node metastasis of BC via bioinformatic analysis

The average expression level of miRNAs was determined based on miR-seq, and 109 miRNAs with an average expression level of above 500 were selected. Among the 1077 BC samples, Pearson's correlation analysis was performed to analyze correlations between the 109 miRNAs and the number of metastatic lymph nodes, to calculate R and P values. miRNAs, with P values below 0.05, were sequenced

based on *R* values. Thus, 19 miRNAs were found to be correlated with lymph node metastasis. Receiver operating characteristic (ROC) analysis was performed on the 19 miRNAs to measure and verify their expression levels for the final purpose of identifying the specificity and sensitivity of lymph node metastasis.

2.3 Evaluation of correlations between expression levels of miRNAs and prognosis

The differential expression of 19 miRNAs related to lymph node metastasis between carcinoma and paracancerous tissues of different samples was determined by testing a negative binomial distribution using the software package edgeR. They were sequenced from the lowest to the highest based on the log fold change (logFC). Expression was deemed to differ significantly if the logFC was above 1 and *P* was below 0.05.

Correlations between expression levels of candidate miRNAs related to lymph node metastasis (including miR-143, miR-455, and miR-99a) and the overall survival (OS) of patients were analyzed by the Kaplan-Meier model. A total of 1077 BC samples were divided into a high-expression group (the upper quartile) and a low-expression group (the later quartile) according to the expression levels of these miRNAs in these samples, to calculate log-rank *P* values and draw survival curves. The relative expression levels of candidate miRNAs related to lymph node metastasis in frozen tissue samples of patients with Her-2-positive infiltrating ductal carcinoma collected by the Zhejiang Cancer Hospital, Hangzhou, China were measured by qRT-PCR and internal controls were normalized. Subsequently, the relationship between the relative expression levels and OS was tested by Pearson's correlation analysis.

2.4 Collection of clinical BC samples

Frozen tissue samples of patients with Her-2-positive infiltrating ductal carcinoma were extracted from the tumor tissue bank of our hospital. Four Her-2-positive BC patients without metastatic lymph nodes, four patients with no more than three metastatic lymph nodes and four patients with more than three metastatic lymph nodes and undergoing radical surgeries for BC from February 2011 to November 2012, were enrolled from our hospital in this study. We collected samples of cancerous and paracancerous tissues from above Her-2-positive BC patients, which were stored at low temperature in the sample bank of our hospital. Patients had been followed up by phone and through visits to outpatient clinics by December 2018. All patients were between 43 and 70 years old and their average age was 55.8 years. No patients enrolled in this study received chemotherapy or radiotherapy before their operations.

2.5 qRT-PCR detection, cell counting kit-8 assay, and transwell cell migration assay

The detection process was performed according to the method of Zhang et al. (2018). The sequences of PCR primers are shown in Table S1.

2.6 Cell culture and miRNA transfection

The cell line MDA-MB-453 was purchased from Shanghai Fuxiang Biological Technology Co., Ltd., China. Cells were cultured on complete Roswell Park Memorial Institute (RPMI) 1640 medium (Hyclone, UT, USA) under normoxic conditions. miRNA transfection was performed using cells in the logarithmic phase (confluence 50% to 80%). The miRNA mimics, miRNA inhibitor, and miRNA control were synthesized by Shanghai GenePharma Co., Ltd. (for sequences of miRNA mimics, refer to Table S2). Transfection assays were performed according to the specifications of the Lipofectamine 2000 reagent kit (Invitrogen, CA, USA). Forty-eight hours after transfection, cells were collected for qRT-PCR to measure the expression of miRNA and *MALAT1*. To test their proliferation and migration, cells were collected using cell counting kit-8 (CCK-8).

2.7 Data analysis

All data were analyzed using SPSS 23.0 software and GraphPad Prism 6.0 (mapping software). The differences in expression of certain genes between the two groups were measured by Student's *t* test. Pearson's correlation coefficients were calculated to analyze the relationship between miRNA expression levels and prognostic factors such as pathological stage, degree of lymph node metastasis, OS, and relapse-free survival (RFS). The data were also analyzed using the Kaplan-Meier model. Two-tailed tests suggested that differences would be significant if the *P* value was <0.05.

3 Results

3.1 Corrections between miR-143, miR-455, miR-99a and lymph node metastasis of BC

To identify genes related to lymph node metastasis, we searched the miR-seq results related to the sampled cancerous and paracancerous tissues using the keyword "BRCA" in the TCGA database, from which miR-seq results and corresponding information of about 1181 samples (including 1077 sampled cancerous and paracancerous tissues of BC) were downloaded. We analyzed correlations between miRNA expression levels and the number of metastatic lymph nodes in different samples. The results showed that 19 miRNAs were correlated to the number of metastatic lymph nodes ($P<0.05$), and these were sequenced according to the R values of the correlations (Table 1).

The ROC, which is commonly used as classifier, accurately reflects the sensitivity and specificity of miRNAs used as markers for diagnosis and prognostic tests. To further narrow the research scope, we built ROC models to analyze the 19 miRNAs to identify their sensitivity and specificity to lymph node metastasis of BC. As the pathological tumor-node-metastasis (pTNM) stage is closely correlated to the number of metastatic lymph nodes and is an important indicator forming the basis for the clinical diagnosis of BC, ROC curves of the 19 miRNAs were analyzed and drawn to verify the correlations. To analyze the correlations between the number of metastatic lymph nodes and the stage (above IIB) based on the ROC, miRNAs such as miR-143, miR-455, and miR-99a were found to cover the largest areas under the curve (AUCs), as shown in Tables 2–4 and Fig. 1. Conjoint analysis was performed on the expression levels of miRNAs. The results suggested that miR-143 and miR-99a occupied the largest AUC in identifying correlations between the number of metastatic lymph nodes and the pathological stage (Tables 2 and 3, Fig. 1). Above all, pure miR-455 or miR-143 and miR-99a might be useful as molecular markers for forecasting the pTNM stage and degree of lymph node metastasis.

3.2 Correlations between miR-455 and survival/prognosis of BC

To analyze how the miRNAs were correlated with the survival and prognosis of BC, we first carried out negative binomial distribution tests and difference analysis on the expression levels of the 19 miRNAs in cancerous and paracancerous tissues, to calculate the logFC for differences in expression levels (Table 1). The results suggested that miR-143 and miR-99a were highly expressed in paracancerous tissues, but poorly expressed in cancerous tissues. The expression levels of these two miRNAs were respectively 1.17 and 2.05 times higher in paracancerous tissues than in cancerous tissues. The expression level of miR-455 was high in paracancerous tissues, but low in cancerous tissues, and the difference was as high as 1.18-fold. Only the expression level of miR-455 was found to be significantly correlated to survival and prognosis after the corresponding correlation analysis was performed using the Kaplan-Meier model. According to the results, prognosis was better in the group where the expression level of miR-455 was higher (Fig. 2). In our study, we proved that a higher expression level of miR-455 in cancerous tissues was associated with fewer lymph nodes and a better prognosis.

To further verify how miR-143, miR-455, and miR-99a in sampled Her-2-positive BC tissues were correlated to lymph node metastasis, prognosis and survival of BC, we collected four Her-2-positive BC samples without lymph node metastasis, four cases with no more than three metastatic lymph nodes and four cases with more than three metastatic lymph nodes to measure the expression levels of miR-143, miR-455, and miR-99a in these samples via qRT-PCR. All patients performed radical surgeries for BC from February 2011 to November 2012. The results suggested that the expression level of miR-455 in the group with a few metastatic lymph nodes was significantly lower than that in the group without lymph node metastasis ($P<0.05$; Fig. 3). Unlike in paracancerous tissues, we did not notice a significant increase in the expression level of miR-455 in cancerous tissues (Fig. 3). In addition, no significant increase was detected in the relative expression level of miR-143 or miR-99a compared with samples without lymph node metastasis or with only a few metastatic lymph nodes. On average, the expression levels of miR-143 and miR-99a were significantly higher in paracancerous tissues than in the group without lymph node metastasis ($P<0.05$; Fig. 3). The relationships between the relative expression levels of miR-143, miR-455 and miR-99a and survivals (including OS and RFS) and

Table 1 Analysis of the correlation between the expression of miRNAs and lymph node metastasis

Gene symbol	logCPM	*P*	*R*	logFC (cancer vs. para-cancer)	Reference	Oncogene or tumor suppressor	Reported as breast cancer metastasis biomarkers
Hsa-miR-542	8.0748	0.0012	0.1068	0.1965	He et al., 2014, 2015; Venkatadri et al., 2016	Tumor suppressor	Controversial
Hsa-miR-10b	16.4057	0.0026	0.0993	−1.9257	Singh et al., 2014; M'hamed et al., 2015; Wang N et al., 2016	Oncogene	Positive correlation
Hsa-miR-99a	9.8641	0.0043	0.0943	−2.0557	Wang et al., 2015; Xia et al., 2016	Tumor suppressor	Inverse correlation
Hsa-miR-143	15.5453	0.0066	0.0898	−1.1712	Smeets et al., 2011; Ng et al., 2014; Li DG et al., 2015; Zhou et al., 2017	Tumor suppressor	Inverse correlation
Hsa-miR-151	11.2302	0.0077	0.0881	0.5065	Krell et al., 2012; Yeh et al., 2016	Tumor suppressor	Inverse correlation
Hsa-miR-193a	9.0143	0.0081	0.0875	−1.1926	Pronina et al., 2017	Tumor suppressor	Not yet reported
Hsa-miR-625	8.0756	0.0179	0.0783	0.3222	Zhou et al., 2016	Tumor suppressor	Not yet reported
Hsa-miR-149	7.4347	0.0183	0.0780	1.1149	Chan et al., 2014; Dong et al., 2017	Tumor suppressor	Not yet reported
Hsa-let-7c	11.5462	0.0270	0.0731	−1.7128	Li XX et al., 2015; Sun et al., 2016; Fu et al., 2017	Tumor suppressor	Not yet reported
Hsa-miR-182	15.4684	0.0288	0.0723	2.2852	Lei et al., 2014; Chiang et al., 2016; Zhan et al., 2017	Oncogene	Positive correlation
Hsa-miR-142	11.5267	0.0289	−0.0722	1.9148	Wang et al., 2014; Schwickert et al., 2015; Cao et al., 2016; Jia et al., 2018	Tumor suppressor	Controversial
Hsa-miR-455	8.1890	0.0319	−0.0709	1.1797	Liu et al., 2016; Wang B et al., 2017	Tumor suppressor	Inverse correlation
Hsa-miR-21	17.8459	0.0328	0.0706	2.1131	Pan et al., 2014; Zhang CF et al., 2016; Wang JL et al., 2017	Oncogene	Positive correlation
Hsa-let-7f-2	12.8123	0.0346	0.0699	−0.0030	Sakurai et al., 2012	Tumor suppressor	Not yet reported
Hsa-miR-25	13.3016	0.0396	0.0680	0.0233	Chang et al., 2016; Asaduzzaman et al., 2017	Oncogene	Not yet reported
Hsa-miR-26a-2	10.9111	0.0416	0.0674	−0.4407	Gao et al., 2013; Liu et al., 2015; M'hamed et al., 2015	Tumor suppressor	Inverse correlation
Hsa-miR-16-1	9.5544	0.0426	−0.0670	0.4870	Patel et al., 2016; Rinnerthaler et al., 2016	Tumor suppressor	Inverse correlation
Hsa-miR-181a-2	9.5920	0.0451	0.0663	0.0798	Guo and Zhang, 2012; Taylor et al., 2013; Wang et al., 2014; Li YY et al., 2015	Controversial	Inverse correlation
Hsa-miR-30d	13.0231	0.0475	0.0655	0.0295	Han et al., 2016; Zhang S et al., 2016	To be determined	Not yet reported

logCPM: logarithm of counts per million reads; logFC: logarithm of fold change

Table 2 ROC analysis of miRNA expression in differentiating lymph node metastasis

Gene symbol	AUC	95% CI	SEN (0.5)	1−SPE (0.5)	*P* value
miR-455	0.544	0.506–0.582	0.545	0.482	0.023
miR-99a	0.586	0.501–0.671	0.620	0.503	0.040
miR-625	0.538	0.501–0.576	0.535	0.487	0.045
miR-16-1	0.538	0.501–0.576	0.539	0.470	0.046
miR-143, miR-455, or let-7c	0.563	0.526–0.600	0.554	0.456	0.001
miR-455 or miR-99a	0.562	0.525–0.600	0.556	0.467	0.001
miR-455, miR-99a, or let-7c	0.562	0.525–0.599	0.557	0.462	0.001
miR-143, miR-455, miR-99a, or let-7c	0.562	0.525–0.600	0.553	0.457	0.001
miR-143 or miR-455	0.561	0.524–0.599	0.555	0.459	0.001
miR-143, miR-455, or miR-99a	0.561	0.524–0.598	0.553	0.459	0.001
miR-455 or let-7c	0.557	0.520–0.595	0.554	0.465	0.003
miR-143+miR-99a or miR-16-1	0.553	0.515–0.591	0.546	0.456	0.007
miR-143+miR-99a	0.613	0.516–0.709	0.658	0.503	0.019
miR-143+miR-99a+miR-625	0.608	0.509–0.706	0.649	0.503	0.026
miR-143+miR-99a+miR-30d	0.601	0.500–0.702	0.639	0.504	0.040
miR-143+miR-99a or miR-625	0.539	0.502–0.577	0.536	0.486	0.041

ROC: receiver operating characteristic; AUC: area under curve; CI: confidence interval; SEN: sensitivity; SPE: specificity; 0.5 is the pathological stage

Table 3 ROC analysis of differentiating early and advanced breast cancers by the expression of miRNAs

Gene symbol	AUC	95% CI	SEN (4.5)	1–SPE (4.5)	*P* value
miR-99a	0.617	0.537–0.697	0.720	0.480	0.005
miR-455	0.550	0.512–0.587	0.530	0.464	0.010
miR-143	0.569	0.515–0.622	0.615	0.472	0.011
miR-30d	0.545	0.507–0.583	0.545	0.458	0.021
Let-7c	0.574	0.507–0.641	0.627	0.480	0.026
miR-143, miR-455, or let-7c	0.583	0.546–0.620	0.556	0.418	0.000
miR-143 or miR-455	0.581	0.544–0.618	0.557	0.422	0.000
miR-143, miR-455, or miR-99a	0.581	0.544–0.618	0.557	0.419	0.000
miR-143, miR-455, miR-99a, or let-7c	0.581	0.544–0.618	0.555	0.418	0.000
miR-455, miR-99a, or let-7c	0.575	0.538–0.612	0.554	0.431	0.000
miR-455 or miR-99a	0.573	0.536–0.610	0.554	0.437	0.000
miR-455 or let-7c	0.573	0.536–0.610	0.552	0.435	0.000
miR-143+miR-99a	0.653	0.566–0.740	0.789	0.480	0.001
miR-143+miR-99a+miR-30d	0.654	0.562–0.745	0.778	0.481	0.002
miR-143+miR-99a+miR-625	0.653	0.564–0.742	0.784	0.481	0.002
miR-143+miR-99a+let-7c	0.638	0.544–0.732	0.765	0.482	0.006
miR-143+let-7c	0.603	0.525–0.681	0.689	0.479	0.007
miR-99a+let-7c	0.614	0.529–0.700	0.714	0.482	0.012
miR-99a or let-7c	0.580	0.515–0.644	0.637	0.477	0.012
miR-143 or miR-99a	0.564	0.512–0.615	0.605	0.471	0.014
miR-99a+miR-182	0.604	0.521–0.687	0.702	0.482	0.016
miR-143+miR-99a or miR-30d	0.546	0.508–0.584	0.547	0.456	0.018
miR-143 or let-7c	0.558	0.508–0.609	0.592	0.472	0.021
miR-143+miR-182	0.562	0.508–0.616	0.608	0.474	0.023
miR-143, miR-99a, or let-7c	0.557	0.507–0.607	0.590	0.472	0.024

ROC: receiver operating characteristic; AUC: area under curve; CI: confidence interval; SEN: sensitivity; SPE: specificity; 0.45 is the pathological stage

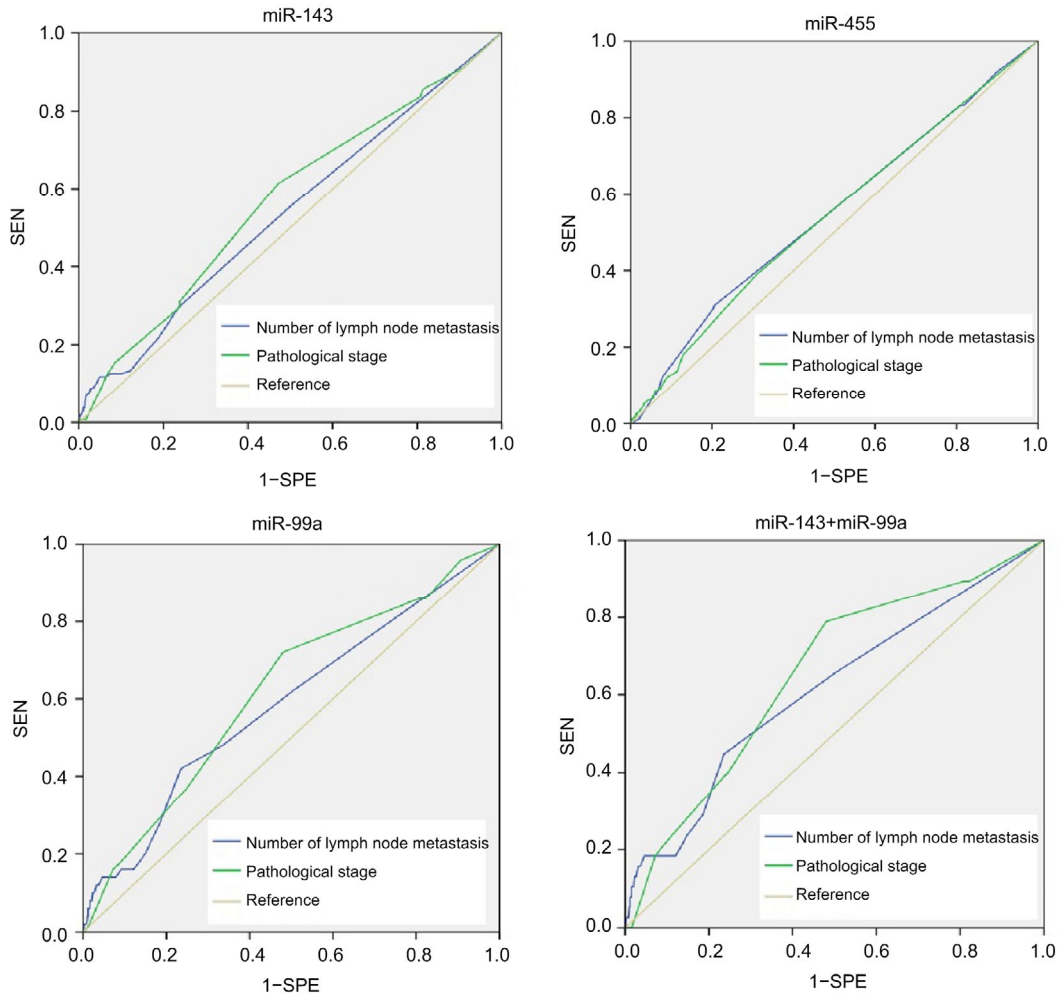

Fig. 1 ROC for distinguishing lymph node metastasis and pathological stages by the expression levels of miRNAs
ROC: receiver operating characteristic; SEN: sensitivity; SPE: specificity

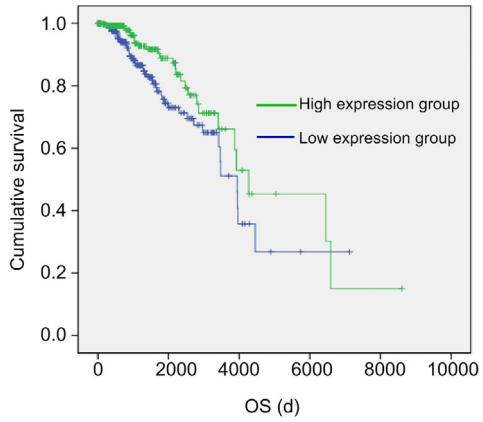

Fig. 2 Correlations between the expression level of miRNA and the survival/prognosis of patients with BC, analyzed by the Kaplan-Meier model
BC: breast cancer; OS: overall survival

prognostic factors (pTNM stage and number of metastatic lymph nodes) were analyzed. Among these three categories of miRNAs, only the expression level of miR-455 was significantly positively correlated with the OS and RFS of patients with Her-2-positive BC (R=0.6394 and R=0.7151, respectively), but there were significant negative correlations with the number of metastatic lymph nodes (R=−0.5769). Nonetheless, the expression levels of all three miRNAs were not correlated with the pTNM stage (Fig. 3, Table 5). These results suggest that the relative expression level of miR-455 could be helpful as a molecular marker for predicting lymph node metastasis and the prognosis of BC. On the other hand, no significant difference existed in the expression level of miR-455 between cancerous and paracancerous tissues of

Table 4 Correlations between the expression of miRNAs and the prognosis and survival of patients

Gene symbol	log-rank *P*
miR-455	0.028
miR-149	0.132
miR-542	0.139
miR-142	0.182
miR-26a	0.228
miR-25	0.315
miR-181a	0.327
miR-21	0.341
miR-625	0.346
miR-99a	0.417
miR-16	0.491
miR-30d	0.538
Let-7c	0.551
miR-143	0.668
miR-193a	0.696
Let-7f	0.722
miR-10b	0.756
miR-182	0.839
miR-151	0.885
miR-143+miR-99a	0.932

Her-2-positive BC. This implies that miR-455 possibly plays crucial roles in lymph node metastasis, but is not critical for other stages of BC, such as the initial stage and advancement.

3.3 Effectiveness of miR-455 for inhibiting cell proliferation and migration in Her-2-positive BC cell lines

To further examine the effects of miR-455 upon proliferation and migration of Her-2-positive BC cells, we studied MDA-MB-453 cells (a kind of Her-2-positive BC cell line) in vitro. The transwell test results suggested that cells became considerably less invasive ($P<0.01$) after the cell line MDA-MB-453 was transfected into miR-455 mimics, while overexpressed miR-455 significantly inhibited proliferation and migration of this cell line (Fig. 4). According to these results, miR-455 plays important roles in the proliferation and migration of Her-2-positive BC cells.

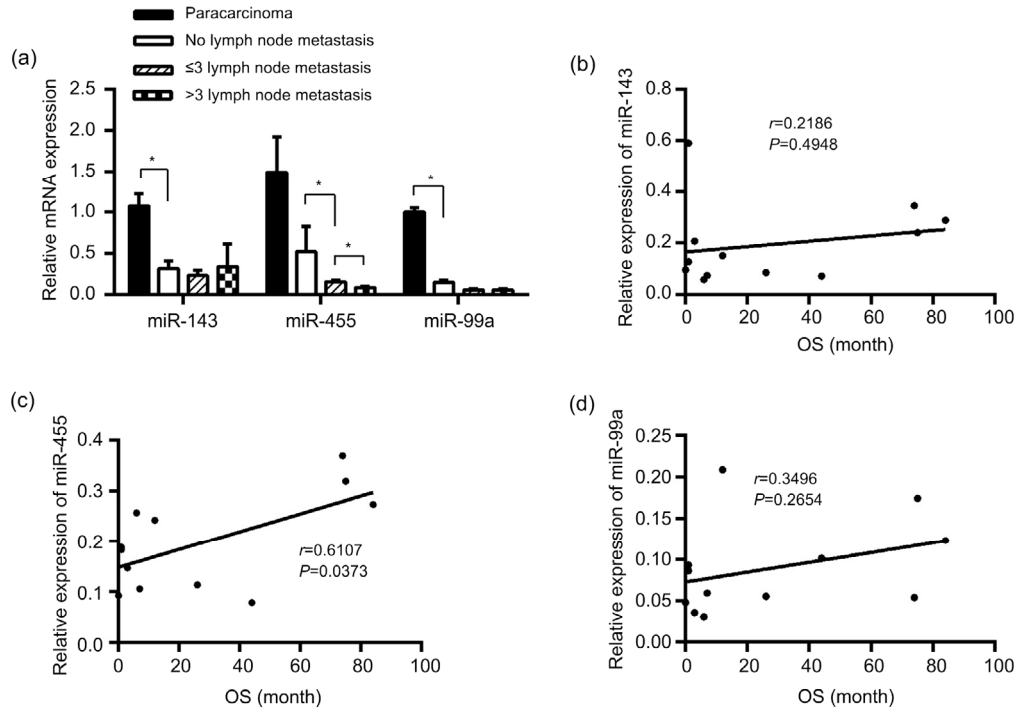

Fig. 3 Correlation analysis between miRNA expression and prognostic survival in Her-2-positive BC cells
(a) Expression levels of miR-143, miR-455, and miR-99a in Her-2-positive BC samples with different degrees of lymph node metastasis were determined by qRT-PCR. The results are represented by the mean±SD. * $P<0.05$. (b–d) Relationships between the expression levels of miR-143 (b), miR-455 (c), and miR-99a (d) in Her-2-positive BC samples and prognosis were analyzed. *r* is Pearson's correlation coefficient. Each data point denotes the ratio between the normalized expression of a patient's internal control and the normalized expression in paracancerous tissues. Her-2: human epidermal growth factor receptor 2; BC: breast cancer; qRT-PCR: quantitative real-time polymerase chain reaction; SD: standard deviation; OS: overall survival

Table 5 Correlation between the expression of miRNAs and prognostic factors in Her-2-positive BC samples

Clinical index	miR-143		miR-455		miR-99a	
	R	*P*	*R*	*P*	*R*	*P*
OS	0.3246	0.3032	0.6394	0.0252	0.4399	0.1524
RFS	0.3515	0.2625	0.7151	0.0089	0.4072	0.1890
Number of lymph node metastases	−0.5554	0.0608	−0.5769	0.0495	−0.3548	0.2578
Pathological stage	−0.0067	0.9836	−0.0981	0.7616	−0.5649	0.0556

Her-2: human epidermal growth factor receptor 2; BC: breast cancer; OS: overall survival; RFS: relapse-free survival

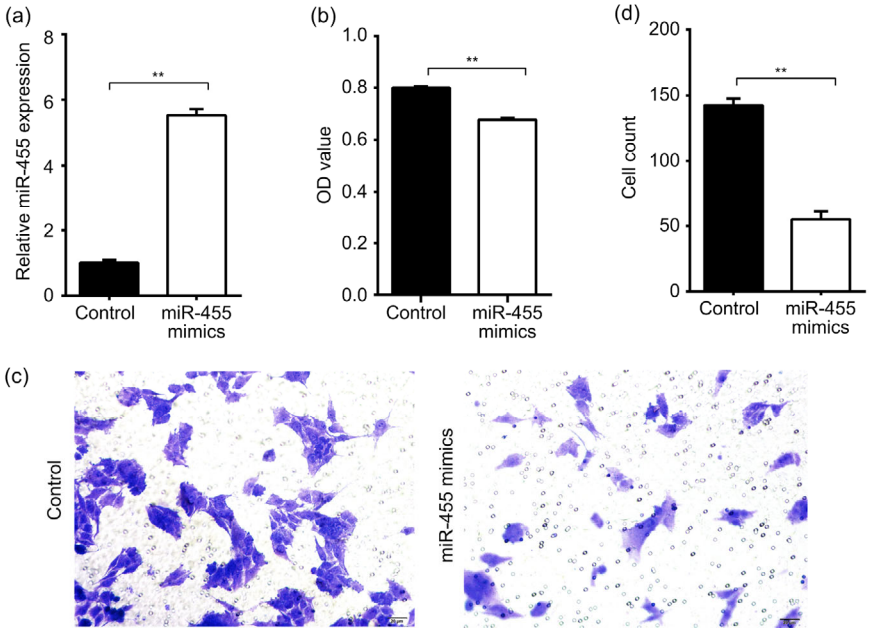

Fig. 4 Effects of overexpressed miR-455 on MDA-MB-453 cell proliferation and invasion

(a) Overexpression of miR-455 in MDA-MB-453 cells after their transfection into miR-455 mimics; (b) Changes to cell proliferation detected by CCK-8 after MDA-MB-453 cell transfection into miR-455 mimics; (c, d) Changes to cell invasiveness by transwell assays after MDA-MB-453 cell transfection into miR-455 mimics. The results are represented by the mean±SD (n=3). ** P<0.01. The scale bar is 20 μm, which has a magnification of 40× under an objective length. CCK-8: cell counting kit-8; SD: standard deviation; OD: optical density

In our previous study, we proved that *MALAT1* is favorable for regulating the proliferation and regulation of Her-2-positive BC cells (Zhang et al., 2018). Likewise, miR-455 also regulates the proliferation and invasion of Her-2-positive BC cells. Thus, we attempted to explore if miR-455 interacts with *MALAT1*. The starBase forecasted that binding bites would exist between *MALAT1* and miR-455 (Fig. 5). qRT-PCR suggested that after transfection of MDA-MB-453 cells into miR-455 mimics, the expression level of *MALAT1* declined significantly (P<0.01; Fig. 5). Finally, we found that miR-455 was effective in inhibiting *MALAT1* expression inside cells. The miR-455 sequence had binding sites for *MALAT1* and overexpressed miR-455 inhibited the expression of *MALAT1*. As it is likely to interact with *MALAT1*, miR-455 plays significant roles in BC proliferation and migration.

4 Discussion

Some miRNAs promote tumor metastasis and advancement, while others play opposite roles (Wang and Luo, 2015). Numerous studies have suggested that lymph node metastasis of BC is closely associated with miRNAs (Chen X et al., 2018; Shiino et al., 2019). High-throughput sequencing and transcriptome analysis are primary options for studying and screening

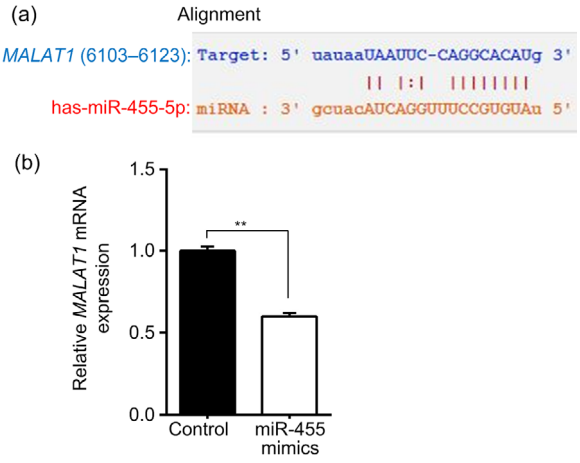

Fig. 5 Effect of overexpressed miR-455 upon the expression level of *MALAT1* in MDA-MB-453 cells
(a) Binding sites between *MALAT1* and miR-455-5p forecasted by starBase; (b) Expression level of *MALAT1* measured by qRT-PCR assay after MDA-MB-453 cell transfection into miR-455 mimics. The results are represented by the mean± SD (n=3). $^{**}P$<0.01. qRT-PCR: quantitative real-time polymerase chain reaction; SD: standard deviation

miRNAs. However, a study with a small sample size is subject to great limitations and systematic deviations. To avoid these shortcomings, we intensively analyzed miR-seq results of 1181 BC samples from the TCGA database, to identify miRNAs related to lymph node metastasis of BC. After analysis, we screened 19 miRNAs related to lymph node metastasis, among which miR-455, miR-143, and miR-99a were found to be highly sensitive and specific for predicting lymph node metastasis. Further analysis suggested that miR-455 was not only related to lymph node metastasis, but also predicted the prognosis of patients. Cytological research showed that miR-455 has biological functions for inhibiting Her-2-positive BC proliferation and migration, which is in line with previous research findings (Wang B et al., 2017; Guo et al., 2018) and further verifies the effectiveness of miR-455 in suppressing cancer. Compared with studies with a small sample size, analysis based on the TCGA is more representative and objective. Above all, our study proved that miR-455, exhibiting low expression in BC tissues, might be used as a molecular marker for forecasting lymph node metastasis and the prognosis of BC.

However, we also noticed that our analysis results conflicted with some previous reports. Among our 19 screened miRNAs related to lymph node metastasis, some miRNAs (including miR-143 and miR-99a) were proven to inhibit BC, although they were positively correlated to lymph node metastasis. This suggests that the expression levels of miRNAs in tumor cells are not necessarily related to their functions. Ng et al. (2014) pointed out that miR-143 exhibited low expression in BC tissues and significantly inhibited cell proliferation once it was overexpressed. Through microarray analysis, Smeets et al. (2011) found that ten kinds of miRNAs, including miR-143, were highly expressed in metastasis-free BC, but exhibited low expression in BC with lymph node metastasis. On the other hand, it was noticed that miR-99a significantly inhibited cell proliferation and migration in triple negative BC cells (Xia et al., 2016). Another microarray study suggested that the expression level of miR-99a was relatively low in BC tissues with lymph node metastasis (Wang et al., 2015). However, the sample sizes in all these studies were small and molecular subtypes were not identified. In this study, for a small amount of Her-2-positive BC samples, the expression levels of miR-143 and miR-99a were found to be relatively low in samples with lymph node metastasis, but analysis of data from the TCGA database suggested that they were high (Table 1, Fig. 3). Therefore, studies with a larger sample size must be carried out to find out if the expression of miR-143 differs among different molecular subtypes of BC. Furthermore, miRNAs might play different roles at different stages of BC. For instance, miR-455 exhibits lower expression in more metastatic BC tissues, whereas its expression level does not differ significantly between poorly metastatic BC tissues and paracancerous tissues (Fig. 3). Analysis of TCGA data suggested that miR-455 even increased slightly in cancerous tissues (Table 1). Therefore, analyzing BC samples at a certain stage is helpful to further identify the expression and biological functions of miRNAs at different stages of BC.

In addition to miRNAs, there is another kind of ncRNA which generally has more than 200 nucleotides in eukaryocytes. These lncRNAs differ slightly from miRNAs in their mechanisms of action, and regulate physiological functions of organisms from multiple perspectives, including epigenetics, transcription, and post-transcription (Vikram et al., 2014). *MALAT1* is a type of conservative lncRNA, highly expressed in

multiple categories of cancers including BC (Jadaliha et al., 2016). However, the molecular mechanism through which *MALAT1* promotes the proliferation and invasion of BC cells is still unknown. According to the study of Chou et al. (2016), *MALAT1* might hinder miRNAs from binding to mRNAs of their target genes by competitively binding to miRNAs as ceRNA inside cells. Our study has proven that miR-455 interacts with *MALAT1*. Nevertheless, more in vitro and in vivo experiments need to be performed to verify the impact of their interactions on their respective biological functions. Moreover, intensive sequencing and bioinformatic analyses need to be carried out to examine the regulatory network of ceRNA and the roles of this gene in BC genesis/advancement in specific cell and tissue environments, although databases including starBase have been used to prove theoretically the interactions between miRNA and lncRNA.

5 Conclusions

According to the TCGA database, we have found that lymph node metastasis of BC could be effectively forecasted according to the expression level of miR-455, because the expression of miR-455 was significantly and positively correlated with the prognosis and OS of BC. In addition, the expression level of miR-455 exhibited a significant positive correlation with the OS and RFS of patients with Her-2-positive BC. It was also significantly and negatively correlated to the number of metastatic lymph nodes in our research. Combining domestic and foreign literature reports, we conclude that miR-455 may be used as a potential factor for forecasting lymph node metastasis and prognosis of Her-2-positive BC.

Contributors

En-qi QIAO participated in writing and revising this paper. Xi-ping ZHANG conceived the idea and wrote this paper. Hong-jian YANG participated in revising the paper. All authors have read and approved the final manuscript and, therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

En-qi QIAO, Hong-jian YANG, and Xi-ping ZHANG declare that they have no conflict of interest.

The approval of the Ethics Committee of Zhejiang Cancer Hospital (Hangzhou, China) was secured for our reported research, and all authors abided by the relevant rules of the Ethics Committee when this study proceeded. The Ethics Committee of Zhejiang Cancer Hospital approved publication of this paper. The research involving human subjects, human material, and human data was performed in accordance with the Declaration of Helsinki 2008 (5) and was approved by an appropriate ethics committee of Zhejiang Cancer Hospital, Hangzhou, China.

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List of electronic supplementary materials

Table S1 Primer information of qRT-PCR
Table S2 Sequence information of miRNA mimics

中文概要

题　目：Her-2 阳性乳腺癌淋巴结转移相关的 miRNA 的筛选及其与患者预后关系的研究

目　的：寻找一种或多种能预测人类表皮生长因子受体 2（Her-2）阳性乳腺癌患者是否发生淋巴结转移及其预后的分子标志物。

创新点：本研究发现，miR-455 与 Her-2 阳性乳腺癌转移相关，可能是一个预测 Her-2 阳性乳腺癌患者淋巴结转移和预后的分子标志物。miR-455 可以通过与长链非编码 RNA 人肺腺癌转移相关转录本 1（*MALAT1*）的相互作用，在乳腺癌的淋巴结转移过程中发挥重要功能。

方　法：通过下载肿瘤基因组图谱（TCGA）数据库中与乳腺癌相关的微小 RNA（miRNA）测序数据，筛选与乳腺癌淋巴结转移相关的 miRNA，进一步分析这些 miRNA 与乳腺癌患者预后的相关性。同时，用实时荧光定量聚合酶链反应（qRT-PCR）方法检测这些 miRNA 在不同程度淋巴结转移的 Her-2 阳性乳腺癌患者组织中的表达水平，及其与预后的相关性。通过细胞学实验研究过表达 miR-455 对 Her-2 阳性乳腺癌细胞系 MDA-MB-453 增殖和侵袭能力的影响，并用 qRT-PCR 检测过表达 miR-455 对 *MALAT1* 表达的影响。

结　论：miR-455 可能是 Her-2 阳性乳腺癌患者淋巴结转移和预后的潜在预测因子。

关键词：乳腺癌；人表皮生长因子受体 2（Her-2）；淋巴结转移；微小 RNA（miRNA）；人肺腺癌转移相关转录本 1（*MALAT1*）